IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Atty Docket No. HYB-004US1)

In Re Application of:	Sudhir Agrawal	Group:	1623
Serial No.:	09/845,623	Examiner:	McIntosh, T.C.
Filed:	April 30, 2001		
Entitled:	Modulation of Oligonucleotide Cpg-Mediated Immunostimulation by Positional Modification of Nucleosides		

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Hon. Assistant Commissioner for Patents:

I, Ekambar Kandimalla, Ph.D., hereby declare as follows:

1. I currently hold the position of Senior Director at Hybridon, Inc. ("Hybridon"). My professional experience, educational background, professional activities, and publications are detailed in the *curriculum vita* which is attached as Exhibit 1.

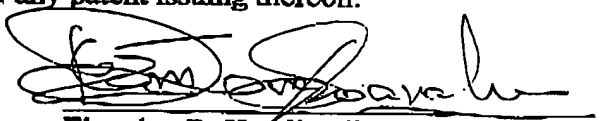
2. I have read the Examiner's rejection of the currently pending claims under 35 U.S.C. §112, first paragraph (as found in the Office Action mailed from the United States Patent and Trademark Office (USPTO) on November 19, 2003), in which the Examiner states that the specification is only enabling for inducing an immune response in a mammal by administering a CpG dinucleotide and an immunomodulatory moiety wherein the immunomodulatory moiety is an abasic nucleoside or a phosphorothioate linkage.

3. Exhibits 2-3, respectively, demonstrate the increased immunostimulatory activity of phosphorothioate oligonucleotides containing CpG motifs in which the deoxynucleosides in the flanking region of the CpG motif are substituted with immunomodulatory moieties, or the substitution of internucleoside linkages with 1,3-propanediol (Exhibit 3) or nitropyrrol and deoxyuridine (Exhibit 2). Taken together with the data demonstrating the ability of abasic nucleoside to increase the immunostimulatory activity, one skilled in the art would expect that the further claimed modifications would also increase the immunostimulatory effect.

4. Additionally, I have read the Examiner's rejection of the currently pending Claim 18 under 35 U.S.C. §112, Second paragraph, in which the Examiner states that it is unclear as to what is intended by the recitation of "P-base".

5. Lin *et al.*, Synthesis of oligodeoxyribonucleotides containing degenerate bases and their use as primers in the polymerase chain reaction, Nucleic Acids Research, 20(19): 5149-5152 (1992), attached as Exbibit 4, shows the structure for 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one, commonly referred to as P-base (see abstract and Figure 1). One skilled in the art would understand the term P-base and the structure it referred to at the time the application was filed.

6. I hereby further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: 

Ekambar R. Kandimalla, Ph.D.

Dated: Apr. 16, 2004

Exhibit 1

EKAMBAR R. KANDIMALLA
Hybrid n, Inc.
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Employment

06/2003 – present	Senior Director of Research, Hybridon, Inc.
08/1999 – 06/2003	Director of Antisense and Functional Genomics, Hybridon, Inc. Application of antisense technology for functional genomics - Antisense oligonucleotide design, synthesis and target validation, fluorescence based PCR probes and primers, CpG-oligodeoxynucleotide-based immunotherapeutics, preclinical studies of antisense oligos.
07/1993 - 07/1999	Sr. Research Scientist, Hybridon, Inc. Design, synthesis, biophysical and biochemical studies of modified antisense and triplex-forming oligos; Studies of the interaction of oligos with biological macromolecules; Solid phase attachment of oligos for diagnostic and analytical uses.
06/1992 - 06/1993	Research Scientist, Hybridon, Inc. Design, synthesis, biophysical and biochemical studies of modified antisense oligonucleotides
09/1987 - 06/1992	Research Associate, Department of Chemistry, University of Alberta. Molecular recognition of nucleic acids; Design, and synthesis of sequence specific minor groove binding peptide antibiotics as anticancer and gene expression control agents; Structural aspects of modified RNA and DNA oligonucleotides; Biophysical, biochemical and molecular biological studies on DNA-binding agents and proteins.
01/1985 - 09/1987	Research Associate, Molecular Biophysics Unit, Indian Institute of Science. Design, synthesis and nucleic acid binding studies of new analogs of DNA binding peptide antibiotics netropsin and distamycin.
02/1981 - 12/1984	Jr. and Sr. Research Fellow, School of Chemistry, Andhra University. Graduate student.

Education

Ph.D.	Chemistry, Andhra University, India	1984
M.Sc.	Biochemistry, Andhra University, India	1980
B.Sc.	Chemistry (Major) & Botony and Zoology, Andhra University, India	1978

Selected Research Publications

83. *FG.Zhu, ER.Kandimalla, D.Yu, JX.Tang*. Modulation of ovalbumin induced Th2 responses by second generation immunomodulatory oligonucleotides in mice. *Int. Immunopharmacol.* 2004 (in press).
82. *ER.Kandimalla & S.Agrawal*. Agonists of Toll-like receptor 9. Modulation of host immune responses with synthetic oligodeoxynucleotides. In Toll-receptors (T.Rich) pp Landes, Cambridge, UK. 2004 (in press).
81. *ER.Kandimalla, RK.Pandey & S.Agrawal*. Hybridization-based fluorescence assay allows quantitation of single-stranded oligodeoxynucleotides in low nanomolar range. *Anal. Biochem.* **328**, 93-95, 2004.

80. *DK.Agrawal, J.Edwan, **ER.Kandimalla**, D.Yu, L.Bhagat, D.Wang & S.Agrawal.* Novel Immunomodulatory oligonucleotides (IMOs) prevent development of allergic airway inflammation and airway hyperresponsiveness in Asthma. *Int. Immunopharmacol.* **4**, 127-138, 2004.
79. D.Wang. Y.Li, D.Yu, S.S.Song. ***ER.Kandimalla*** & S.Agrawal. Immunopharmacological and antitumor effects of second-generation immunomodulatory oligonucleotides containing synthetic CpR motifs. *Int. J. Oncol.* **24**. 901-908, 2004 .
78. *S.Agrawal & **ER.Kandimalla**.* Modulation of Toll-like receptor 9 responses through synthetic immunostimulatory motifs of DNA. *Ann. N. Y. Acad. Sci.*, **1002**, 30-42, 2003.
77. ***ER.Kandimalla** & S.Agrawal.* Chemistry of CpG DNA. In *Curr. Prot. Nucleic Acids Chem.* (Ed. S. Beucauge), pp 4.13.1-4.13.13, John-Wiley, New York, 2003.
76. ***ER.Kandimalla***, L.Bhagat. FG.Zhu, D.Yu, YP.Cong, D.Wang, JX.Tang, JY.Tang, CF.Knetter, E.Lien & S.Agrawal. A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and overrides species specific recognition observed with CpG motif. *Proc. Natl. Acad. Sci. USA.* **100**, 14303-14308, 2003.
75. YP.Cong, SS.Song, L.Bhagat, RK.Pandey, D.Yu, ***ER.Kandimalla*** & S.Agrawal. Self-stabilized CpG DNAs optimally activate human B cells and plasmacytoid dendritic cells. *Biochem. Biophys. Res. Commun.* **310**, 1133-1139, 2003.
74. ***ER.Kandimalla***, L.Bhagat, YP.Cong, RK.Pandey, D.Yu, Q.Zhao & S.Agrawal. Secondary structures in CpG oligonucleotides affect immunostimulatory activity. *Biochem. Biophys. Res. Commun.* **306**, 948-953, 2003.
73. ***ER.Kandimalla***, L.Bhagat, D.Wang, D.Yu, FG.Zhu, J.Tang, H.Wang, P.Huang, R.Zhang & S.Agrawal. Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles. *Nucleic Acids Res.*, **31**, 2393-2400. 2003.
72. D.Yu, ***ER.Kandimalla***, Q.Zhao, L.Bhagat, Y.Cong & S.Agrawal. Requirement of nucleobase proximal to CpG dinucleotide for immunostimulatory activity of synthetic CpG DNA. *Bioorg. Med. Chem.* **11**, 459-464, 2003.
71. ***ER.Kandimalla**, FG.Zhu, L.Bhagat, D.Yu & S.Agarawal.* Toll-like receptor 9: Modulation of recognition and cytokine induction by novel synthetic CpG DNAs. *Biochem. Soc. Trans.*, **31**. 654-658, 2003.
70. L.Bhagat, F.G.Zhu, D.Yu, J.Tang, H.Wang, E.R.Kandimalla, R.Zhang, S.Agrawal. CpG Penta- and Hexadeoxyribonucleotides as Potent Immunomodulatory Agents. Biochem. Biophys. Res. Commun. 300 (2003) 853-861.
69. D.Yu. ***ER.Kandimalla***, L.Bhagat, JY.Tang, Y.Cong, J.Tang & S.Agrawal. 'Immunomers' - Novel 3'-3'-linked CpG oligodeoxynucleotides as potent immunomodulatory agents. *Nucleic Acids Res.*, **30**, 4460-4469, 2002.
68. D.Yu, F-G.Zhu. L.Bhagat, H.Wang, ***ER.Kandimalla***, R.Zhang & S.Agrawal. Potent CpG oligonucleotides containing phosphodiester linkages: In vitro and in vivo immunostimulatory properties. *Biochem. Biophys. Res. Commun.* **297**, 83-90, 2002.
67. ***ER.Kandimalla***, L.Bhagat, D.Yu, Y.Cong, J.Tang & S.Agrawal. Conjugation of ligands at the 5'-end of CpG DNA affects immunostimulatory activity. *Bioconj. Chem.* **13**, 966-974, 2002.
66. D.Yu, ***ER.Kandimalla***, Y.Cong, J.Tang, JY.Tang, Q.Zhao & S.Agrawal. Design, synthesis, and immunostimulatory properties of CpG DNAs containing alkyl-linker substitutions: Role of nucleosides in the flanking sequences. *J. Med. Chem.* **45**, 4540-4548, 2002.
65. D.Yu, ***ER.Kandimalla***, Q.Zhao, Y.Cong & S.Agrawal. Immunostimulatory properties of phosphorothioate CpG DNA containing both 3'-5'- and 2'-5'-internucleotide linkages. *Nucleic Acids Res.* **30**, 1613-1619, 2002.
64. *H.Wang, J.Hang, Z.Shi, M.Li, D.Yu, **ER.Kandimalla**, S.Agrawal & R.Zhang.* Antisense oligonucleotide targeted to RIα subunit of cAMP-dependent protein kinase (GEM 231) enhances therapeutic effectiveness of cancer chemotherapeutic agent irinotecan in nude mice bearing human cancer xenografts: *In vivo* synergistic activity, pharmacokinetics and host toxicity. *Int. J. Oncol.* **21**, 73-80, 2002.
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62. ***ER.Kandimalla***, *D.Yu*, *& S.Agrawal*. Towards Optimal Design of Second-Generation Immunomodulatory Oligonucleotides. *Curr. Opin. Mol. Ther.*, **4**, 122-129, 2002
61. *S.Agrawal & **ER.Kandimalla***. Medicinal chemistry and therapeutic potential of CpG-DNA. *Trend. Mol. Med.*, **8**, 114-121, 2002.
60. *BJ.Premraj, PK.Patel., **ER.Kandimalla**, S.Agrawal, RV.Hosur & N.Yathindra*. NMR Structure of a 2'-5' RNA favors A-type duplex with compact C2'-endo nucleoside repeat. *Biochem. Biophys. Res. Commun.* **283**, 537-543, 2001.
59. *S.Agrawal & **ER.Kandimalla***. Antisense and/or immunostimulatory oligonucleotide therapeutics. Curr. Cancer Drug Targets, **1**, 197-209, 2001.
58. ***ER.Kandimalla** & S.Agrawal*. Therapeutic Potential of Synthetic CpG DNA-Current Status and Future Directions. I. Drugs, **4**, 963-966, 2001.
57. D.Yu, ***ER.Kandimalla***, Q.Zhao. Y.Cong & S.Agrawal. Modulation of immunostimulatory activity of CpG oligonucleotides by site-specific deletion of nucleobases. *Booorg. Med. Chem. Lett.* **11**, 2263-2267, 2001.
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55. ***ER.Kandimalla***, D.Yu, Q.Zhao & S.Agrawal. Effect of chemical modifications of cytosine and guanine in a CpG-motif of oligonucleotides on immunostimulatory activity: Structure-immunostimulatory activity relationships. *Bioorg. Med. Chem.* **9**, 807-813, 2001.
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53. *SL.Shankar, S.Mani, KN.O'Guin, **ER.Kandimalla**, S.Agrawal & B.Shafit-Zagardo*. Survivin inhibition induces human neural tumor cell death through caspase-indepenent and -dependent pathways. *J. Neurochem.* **79**, 426-436, 2001.
52. *F.Ciardiello, R.Caputo, T.Troiani, **ER.Kandimalla**, S.Agrawal, J.Mendelsohn, AR.Bianco & G.Tortora*. Antisense oligonucleotides targeting the epidermal growth factor receptor inhibit proliferation, induce apoptosis, and cooperate with cytotoxic drugs in human cancer cell lines. *Int. J. Cancer* **93**, 172-178, 2001.
51. *Y.Lu, S.Mani, **ER.Kandimalla**, D.Yu, S.Agrawal, JC.States & DB.Bregman*. The cockayne syndrome group B DNA repair protein as an anti-cancer target. *Int. J. Oncol* **19**, 1089-1097, 2001.
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49. *S.Agrawal & **ER.Kandimalla***. Antisense therapeutics. Is it as simple as complementary base recognition? Mol. Med. Today, **6**, 72-81, 2000.
48. *D.Yu, **ER. Kandimalla**, A.Roskey, Q.Zhao, L.Chen, J.Chen & S.Agrawal*. Stereo-enriched phosphorothioate oligodeoxynucleotides: Synthesis, biophysical and biological properties. *Bioorg. Med. Chem.* **8**, 275-284, 2000.
47. ***ER.Kandimalla** & S.Agrawal*. 'Cyclicons' as hybridization-based fluorescent primer-probes - Synthesis, properties and application in real-time PCR. *Bioorg. Med. Chem.* **8**, 1911-1916, 2000.
46. *S.Agrawal & **ER.Kandimalla***. Medicinal chemistry of antisense oligonucleotides. In Antisense Technology in the Central Nervous System, (Eds. R.Leslie, J.Hunter and H.Robertson), pp108-136, Oxford University Press, Oxford, 1999.
45. *Z.Jiang, **ER.Kandimalla**, Q.Zhao, LX.Shen, A.DeLuca, N.Normano, M.Ruskowski & S.Agrawal*. Pseudo-cyclic oligonucleotides: *In vitro* and *in vivo* properties. *Bioorg. Med. Chem.* **7**, 2727-2735, 1999.
44. ***ER.Kandimalla**, DR.Shaw & S.Agrawal*. Effects of phosphorothioate oligodeoxyribonucleotide and oligoribonucleotides on human complement and coagulation. *Bioorg. Med. Chem. Lett.* **8**, 2103-2108, 1998.
43. *S.Agrawal, X.Zhang, Q.Cai, **ER.Kandimalla**, A.Manning, Z.Jiang, T.Marcel & R.Zhang*. Effect of aspirin on protein binding and tissue disposition of oligonucleotide phosphorothioate in rats. *J. Drug Target.* **5**, 303-312, 1998.
42. *LX.Shen, **ER.Kandimalla** & S.Agrawal*. Impact of mixed-backbone oligonucleotides on target binding affinity and target cleaving specificity and selectivity by E. coli RNase H. *Bioorg. Med. Chem.* **6**, 1695-1705, 1998.

41. *DR.Shaw, PK.Rustagi, **ER.Kandimalla**, AN.Manning, Z.Jiang & S.Agrawal.* Effects of synthetic oligonucleotides on human complement and coagulation. *Biochem. Pharmacol.* **53**, 1123-1132,1997.
40. ***ER.Kandimalla**, G.Venkataraman, V.Sasisekharan & S.Agrawal.* Single-stranded DNA and RNA targeted triplex-formation: UV, CD and molecular modeling studies of foldback triplexes containing different RNA and DNA strand combinations. *J. Biomolec. Struc. Dyn.* **14**, 715-726, 1997.
39. ***ER.Kandimalla**, A.Manning, Q.Zhao, DR.Shaw, RA.Byrn, V.Sasisekharan & S.Agrawal.* Mixed backbone antisense oligonucleotides: Design, biochemical and biological properties of oligonucleotides containing 2'-5'-ribo and 3'-5'-deoxyribo-nucleotide segments. *Nucleic Acids Res.* **25**, 370-378, 1997.
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37. ***ER.Kandimalla** & S.Agrawal.* Hoogsteen DNA duplexes of 3'-3' and 5'-5' attached oligonucleotides and triplex-formation with RNA and DNA pyrimidine sequences: Experimental and molecular modeling studies. *Biochemistry* **35**, 15332-15339, 1996.
36. ***ER.Kandimalla**, A.Manning & S.Agrawal.* Single strand targeted triplex formation: Physicochemical and biochemical properties of foldback triplexes. *J. Biomol. Struct. Dyn.* **14**, 79-90, 1996.
35. ***ER.Kandimalla**, A.Manning & S.Agrawal.* Single strand targeted triplex formation: Strand displacement of duplex DNA by foldback triplex-forming oligonucleotides. *J. Biomol. Struct. Dyn.* **13**, 483-492, 1995.
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30. ***ER.Kandimalla** & S.Agrawal.* Destabilization of DNA guanine quadruplex structure by foldback triplex-forming oligodeoxynucleotides. *Nucleosides Nucleotides* **14**, 991-995, 1995.
29. ***ER.Kandimalla**, J.Temsamani & S.Agrawal.* Synthesis and properties of 2'-O-methylribonucleotide methylphosphonate containing chimeric oligonucleotides. *NucleosidesNucleotides* **14**, 1031-1035, 1995.
28. ***ER.Kandimalla** & S.Agrawal.* Single strand targeted triplex formation: Stability, specificity and RNase H activation properties. *Gene* **149**, 115-121, 1994.
27. ***KE.Rao**, G.Gosselin, D.Mrani, C.Perigaud, JL.Imbach, C.Bailly, JP.Henichart, P.Colson, C.Houssier & JW.Lown.* Psoralen-lexitropsin hybrids: DNA sequence selectivity of photoinduced cross-linking from MPE footprinting and exonuclease III stop assay, and mode of binding from electric linear dichroism. *Anticancer Drug Des.* **9**, 221-237, 1994.
26. ***KE.Rao**, S.Padmanabhan & JW.Lown.* Molelcular recognition between ligands and nucleic acids: Sequence preferences and binding of pyrrolo[3,2-d] and [2,3-d]thiazole-containing lexitropsins deduced from MPE.Fe(II) footprinting. *Actual. Chim. Ther.* **20**, 159-188, 1993.
25. *F.Adnet, J.Liquier, E.Taillandier, MP.Singh, **KE.Rao** & JW.Lown.* FTIR study of specific binding interactions between DNA minor groove binding ligands and polynucleotides. *J. Biomol. Struc. Dyn.* **10**, 565-575, 1992.
24. *D.Mrani, G.Gosselin, C.Bailly, R.Houssin, **KE.Rao**, J.Zimmermann, J.Balzarani, E.DeClercq, JP.Henichart, JW.Lown & JL.Imbach.* Synthesis, DNA binding and biological evaluation of bithiazol-linked netropsin derivatives. *Eur. J. Med. Chem.* **27**, 331-344, 1992.
23. ***KE.Rao** & JW.Lown.* Molecular mechanism of action of saframycin antibiotics: Sequence selectivities in the covalent bonding of saframycins Mx1, Mx3, A and S deduced from MPE.Fe(II) footprinting and exonuclease stop assays. *Biochemistry* **31**, 12076-12082, 1992.
22. ***KE.Rao** & JW.Lown.* Lexitropsins: Sequence selective DNA binding and anticancer agents. *Trend. Org. Chem.* **3**, 141-171, 1992.

21. *TA.Beerman, MM.McHugh, R.Sigmund, JW.Lown, **KE.Rao** & Y.Bathini.* Effects of analogs of the DNA minor groove binder Hoechst 33258 on topoisomerase II and I mediated activities. *Biochim. Biophys. Acta* **1131**, 53-61, 1992.
20. ***KE.Rao**, K.Krowicki, G.Burckhardt, C.Zimmer & JW.Lown.* Molecular recognition between oligopeptides and nucleic acids: DNA binding selectivity of a series of 1,2,4-triazole-containing lexitropsins. *Chem. Res. Toxicol.* **4**, 241-252, 1991.
19. ***KE.Rao**.* Synthesis of distamycin and netropsin analogs. Part IV. Synthesis of bis-1,3-[3/4 (guanidinoacetamido) benzamido] benzene dihydrochlorides and bis-1.3-[4/3,5-diaminobenzamido) benzamido] benzene tetrahydrochloride. *Indian J. Chem.* **30B**, 13-17, 1991.
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16. *TA.Beerman, JW.Woynarowski, RD.Sigmund, LS.Gawron, **KE.Rao** & JW.Lown.* Netropsin and bis-netropsin analogs as inhibitors of the catalytic activity of mammalian DNA topoisomerase II and topoisomerase cleavable complexes. *Biochim. Biophys. Acta* **1090**, 52-60, 1991.
15. ***KE.Rao** & JW.Lown.* Molecular recognition between ligands and nucleic acids: DNA binding characteristics of analogues of Hoechst 33258 designed to exhibit altered base and sequence recognition. *Chem. Res. Toxicol.*, **4**, 661-669, 1991.
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13. *SM.Lal, **KE.Rao**, YN.Vaishnav, BU.Rao & V.Sasisekharan.* Antiviral activity and inhibition of macromolecular synthesis of human neoplastic cells by synthetic analogues of distamycin and netropsin. *Indian J. Virol.*, **7**, 1-11. 1991.
12. *WH.Gmeiner, **KE.Rao**, B.Rayner, JL.Imbach & JW.Lown.* Polarity of annealing and structural analysis of the α-5'-d[TACACA]:β-5'-r[AUGUGU] hybrid resistant to RNase H mediated hydrolysis determined by high field ^{1}H, ^{13}C and ^{31}P NMR analysis. *Biochemistry* **29**, 10329-10341, 1990.
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10. *Y.Bathini, **KE.Rao**, RG.Shea & JW.Lown.* Molecular recognition between ligands and nucleic acids: Novel pyridine- and benzoxazole-containing agents related to Hoechst 33258 that exhibit altered DNA sequence specificity deduced from footprinting analysis and spectroscopic studies. *Chem. Res.Toxicol.* **3**, 268-280, 1990.
9. ***KE.Rao** & V.Sasisekharn.* Synthesis of distamycin and netropsin analogs: Part II - DNA binding bisquaternary ammonium heterocycles analogous to NSC 101327. *Indian J. Chem.* **29B**, 503-507, 1990.
8. ***KE.Rao** & V.Sasisekharan.* Synthesis of distamycin and netropsin analogs: Part III - Biologically active analogs of tris(m-benzamido) compound. *Indian J. Chem.* **29B**, 508-513, 1990.
7. ***KE.Rao**, K.Krowicki, J.Balzarini, E.DeClercq, RA.Newman & JW.Lown.* Novel linked antiviral and antitumor agents related to netropsin - 2: Synthesis and biological evaluation. *Actual. Chim. Ther.* **18**, 21-42, 1990.
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3. ***KE.Rao**, N.Ramesh, D.Choudhury, SK.Brahmachari & V.Sasisekharan.* Role of the environment in the interaction of non-intercalators with Z-DNA. *J. Biomol. Struc. Dyn.* **7**, 335-345, 1989.

2. ***KE.Rao, D.Dasgupta & V.Sasisekharan.*** Interaction of synthetic analogues of distamycin and netropsin with nucleic acids. Does curvature of ligand play a role in distamycin-DNA interactions? *Biochemistry,* **27**, 3018-3024, 1988.
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Patents

8. Pseudo-cyclic oligonucleobases (US/6,383,752)
7. Affinity-based purification of oligonucleotides using soluble multimeric oligonucleotides (US/5,912,332).
6. Mixed backbone antisense oligonucleotides conatining 2'-5'-ribonucleotide- and 3'-5'-deoxyribonucleotide segments (US/5,886,165).
5. Triplex-forming antisense oligonucleotides having abasic linkers targeting nucleic acids comprising mixed sequences of purines and pyrimidines (US/5,693,773).
4. Cooperative oligonucleotides (US/6,372,427).
3. Integrated oligonucleotides (US/5,739,308).
2. Oligonucleotide alkylphosphonates and alkylphosphonothioates (EP/677056).
1. Foldback triplex-forming oligonucleotides (EP/680489)

Selected presentations at conferences

Over 25 conference presentations

CpG DNA recognition: Overriding species specificity with novel synthetic motifs. Speaker at Biochemical Society Focused Meeting on Toll-Like Receptors, Novartis, Horsham, UK, February 03, 2003.

Modulating immune responses with oligonucleotides containing synthetic motifs. Chemistry and therapeutic applications. Speaker at SRI's Oligonucleotides in Drug Discovery & Development Conference, Long Branch, NJ, March 4-5, 2002.

Oligonucleotide-based therapeutic agents - Antisense or immune stimulation: The crossroads. Speaker at IBC's Tides 2001 - Oligonucleotide and Peptide Technology Conferences, Tucson, AZ, April 24-27, 2001.

Cyclicons-Fluorescent primer-probes for hybridization-based assays. Speaker at IBC's meeting on Nucleic Acid Detection and Screeing Technologies. State of the art DNA, RNA and PNA-based applications, San Diego, September 25-27, 2000.

Mixed-backbone oligonucleotides: Antisense agents with improved pharmacological properties. Speaker at Cambridge Healthtech Institute's meeting on Antisense Technologies, San Francisco, June 22-23, 1998 (unable to attend).

Mixed backbone antisense oligonucleotides containing 2'-5'-ribo- and 3'-5'-deoxyribonucleosides: Synthesis, biochemical and biological properties. Speaker at 23rd Symposium on Nucleic Acids Chemistry, Gifu, Japan, November 12-14, 1996.

Molecular recognition of nucleic acids: Parallel-stranded Hoogsteen duplexes and triplex formation with RNA/DNA pyrimidine single strands. Speaker at 212th American Chemical Society National Meeting, Medicinal Chemistry, Orlando, FL, August 25-29, 1996 (C&E News, July 22, 1996, pp104).

Foldback triplex-forming oligonucleotides. Physicochemical and biochemical properties. Speaker at 24th ACS North Eastern Regional Meeting, Burlington, VT, June 19-22, 1994 (C&E News, April, 18, 1994, pp52).

Design of antisense oligonucleotides: Single-stranded RNA/DNA targeted triplex-formation. Speaker at Gordon Conference on Purines, Pyrimidines and Related Substances, New Port, RI, July 5-9, 1993.

Figure 1

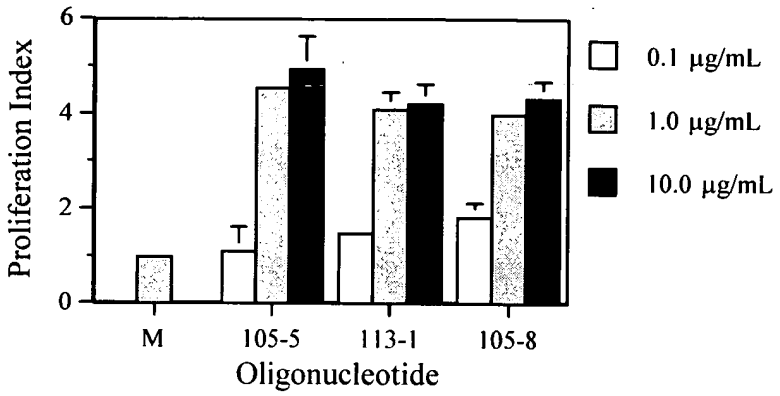
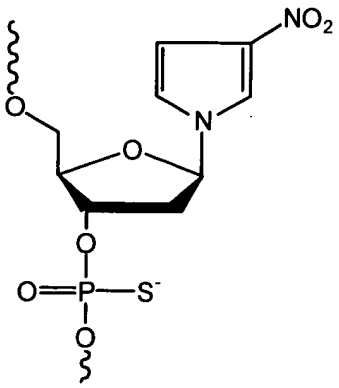


Figure 1. Proliferation of BALB/c mouse spleen cells in cultures incubated for 24 hrs with or without oligonucleotides at different concentrations.

113-1	5'-TCCATGACGTTCCTGATGC-3'
105-5	5'-TCCA**X**GACGTTCCTGATGC-3'
105-8	5'-TCCATGACGTT**X**CTGATGC-3'

All are phosphorothioate oligodeoxynucleotides. X = 2'-deoxyribo-nitropyrrole.



2'-deoxynitropyrrole

Figure 2A

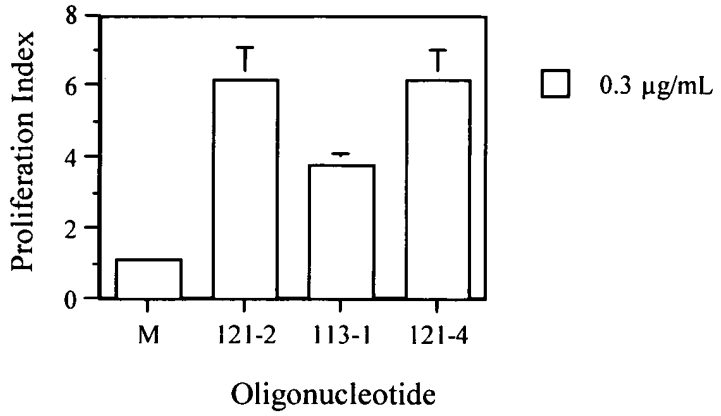


Figure 2A. Proliferation of BALB/c mouse spleen cells in cultures incubated for 24 hrs with or without oligonucleotides at 0.3 µg/ml concentration.

Figure 2B

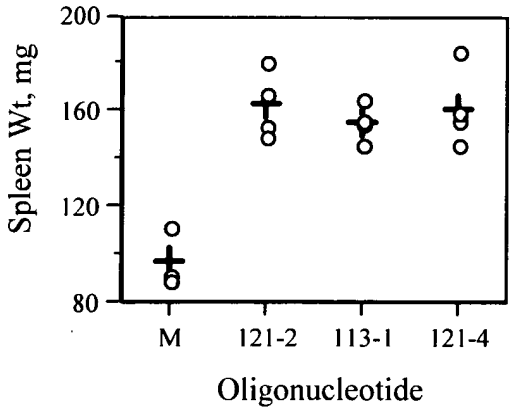
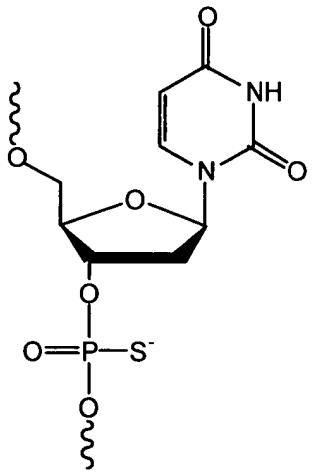


Figure 2B. Spleen enlargement in BALB/c mouse, 72 hr after administration of oligonucleotides at a dose of 5 mg/kg or PBS through i.p. injection.

113-1	5'-TCCATGACGTTCCTGATGC-3'
121-2	5'-TCCAT**Y**ACGTTCCTGATGC-3'
121-4	5'-TCCATGACGT**Y**CCTGATGC-3'

All are phosphorothioate oligodeoxynucleotides. **Y** = 2'-deoxyribo-uridine.



2'-deoxyuridine

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Exhibit 3

J. Med. Chem. **2002**, *45*, 4540−4548

Design, Synthesis, and Immunostimulatory Properties of CpG DNAs Containing Alkyl-Linker Substitutions: Role of Nucleosides in the Flanking Sequences

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Received April 17, 2002

Bacterial and synthetic DNA containing unmethylated CpG dinucleotides activate the innate immune system and promote Th1-like immune responses. Recently, a receptor, TLR9, has been shown to recognize CpG DNA and activate immune cascade. But there have been no reports on the molecular mechanisms of recognition between CpG DNA and the receptor(s). Our earlier studies described a number of the chemical and structural characteristics of CpG dinucleotide and the sequences flanking the CpG dinucleotide that are critical for immunostimulatory activity. In the present study, we examined the effect of the presence and absence of a nucleoside in the flanking sequences by replacing one or two natural deoxyribonucleosides at various positions with one or more alkyl- (C2−C12), branched alkyl- (glyceryl or aminobutyryl-propanediol), or ethyleneglycol- (tri or hexa) linkers. The results suggest that a linker substitution at the first two nucleoside positions adjacent to the CpG dinucleotide on the 5′- or the 3′-side neutralizes the immunostimulatory activity, as determined by in vitro mouse spleen cell proliferation, cytokine secretion, and in vivo mouse spleen enlargement. The same substitutions placed about three to six nucleotides away from the CpG dinucleotide either did not affect or potentiated immunostimulatory activity compared with parent CpG-DNA without modifications. Substitution of deoxyribonucleosides with a C3 or C4 alkyl-linker was found to be optimal for potentiating immunostimulatory activity.

Introduction

The cells of the vertebrate immune system (macrophages, monocytes, dendritic cells, NK cells, and B-cells) recognize unmethylated CpG dinucleotides present in bacterial and synthetic oligodeoxynucleotides (CpG-DNA) in a sequence-specific manner and secrete a myriad of cytokines and chemokines, including IL-12, IL-6, TNF-α, and INF-γ, and up-regulate expression of costimulatory molecules.[1−4] The cytokines secreted provide nonspecific protection against infectious pathogens and strongly enhance the generation of antigen-specific immune responses.[5−9] A number of CpG-DNAs are at various stages of preclinical and clinical evaluation as antitumor, antiviral, antibacterial, and anti-asthmatic agents and as adjuvants in immunotherapy.[6−19]

The presence of a CpG dinucleotide with specific nucleotides in the flanking sequences is critical for the immunostimulatory activity of CpG-DNAs.[3,20,21] Our earlier studies have shown that sugar (ribose or 2′-O-methylribose) or backbone (nonionic methylphosphonate) modifications within a CpG dinucleotide neutralized the immunostimulatory activity.[22] The same modifications distal to the CpG dinucleotide did not neutralize the immunostimulatory activity of CpG DNAs.[23−25] Moreover, the incorporation of these modifications in the 5′-flanking sequence enhanced the immunostimulatory activity of CpG DNA.[23−25] Additionally, our recent studies suggest that an accessible 5′-end of CpG-DNA is critical for immunostimulatory activity.[26]

We have shown that substitution of C or G within a CpG dinucleotide with certain modified pyrimidine or purine bases does not affect the immunostimulatory activity of CpG DNA.[27] We have also shown that site-specific substitution of an anionic internucleoside linkage with a nonionic methylphosphonate linkage also affects the immunostimulatory activity of CpG-DNA.[25] The deletion of a nucleobase at specific sites distal to the CpG dinucleotide enhances the immunostimulatory activity of CpG-DNA.[28] Our recent studies, in which a 3′-deoxyribonucleoside is incorporated for 2′-deoxyribonucleoside in CpG DNA, suggest that structural perturbation caused by 2′-5′-internucleoside linkage at least about 3−5 nucleotides distal to the CpG dinucleotide in the 5′-flanking sequence potentiates immunostimulatory activity.[29] Incorporation of the same modification in the 3′-flanking sequence has minimal effect on immunostimulatory activity.[29] To further understand the significance of nucleosides in the flanking sequences for immunostimulatory activity, we synthesized a series of CpG-DNAs with one or more natural nucleosides substituted with nonnucleoside linkers (Figure 1) and studied their structure-immunostimulatory activity relationships in BALB/c mouse spleen cell cultures and in vivo.

Results and Discussion

Two 18-mer DNA sequences containing either an "AGCGTT" (**10**) or "GACGTT" (**23**) hexameric motif containing a CpG dinucleotide were selected for the present studies (Table 1). Both parent CpG-DNAs **10** and **23** induced immune responses in mouse.[25,28,29] A series of modified CpG-DNAs incorporating one or more

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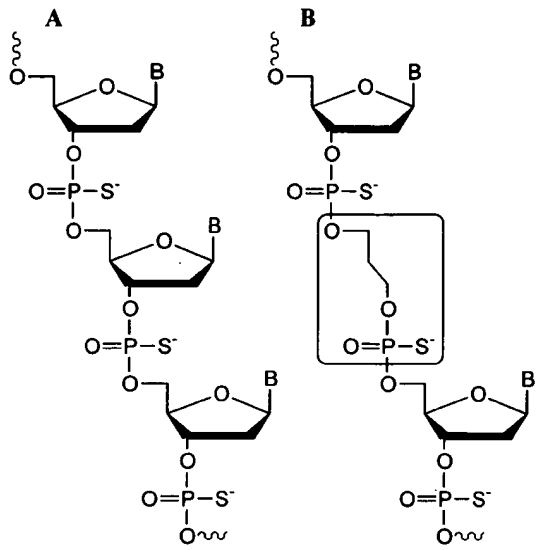

Figure 1. Representative structures of (A) a deoxyribonucleotide chain and (B) a deoxyribonucleotide chain containing a C3-linker (boxed). B stands for A, C, G, or T.

linkers (**1**–**9**) (Figure 2) at various nucleotide positions in parent CpG DNAs. **10**, and **23** (Table 1) were synthesized and studied for their immunostimulatory activity, as determined by spleen cell proliferation and cytokine secretion in BALB/c mouse spleen cell cultures and in vivo spleen enlargement (splenomegaly) in BALB/c mice.

Nucleosides in Certain Positions of CpG DNA Are Not Required for Immunostimulatory Activity. To study the effect of deletion of a nucleoside, a propanediol- (C3) linker, **2** (Figure 2), was incorporated in place of a 2′-deoxynucleoside at different positions in the 5′- and the 3′-flanking sequences and within the CpG dinucleotide of the parent CpG DNA **10** (CpG DNAs **11**–**18**; Table 1). The incorporation of propanediol-linker (**2**) allowed maintenance of appropriate distance between two adjacent phosphates similar to that found in oligodeoxyribonucleotides (Figure 1). Spleen cells obtained from BALB/c mice were cultured with CpG-DNAs at 0.1, 0.3, 1.0, and 3.0 μg/mL concentrations for 48 h, and the cell proliferation was measured by ^{3}H-uridine incorporation.[22,30] All CpG DNAs that were active showed a concentration-dependent spleen cell proliferation. The extent of spleen cell proliferation induced at 0.3 μg/mL concentration of CpG DNAs is shown in Table 2 as proliferation index (PI). The parent CpG-DNA, **10**, induced significant spleen cell proliferation (1.7 ± 0.27) compared with media (PBS) control (0.2 ± 0.06). CpG-DNA **11**, which had a C3-linker (**2**) substituted for G in CpG dinucleotide, induced spleen cell proliferation similar to that of media control. Consistent with spleen cell proliferation data, CpG DNA **10** induced cytokines IL-12 and IL-6 in a concentration-dependent manner in BALB/c mouse spleen cell cultures (Supplementary Table 1). CpG DNA **10** induced 1391 ± 179 pg/mL of IL-12 and 6644 ± 380 pg/mL of IL-6 secretion at 1.0 μg/mL concentration. CpG DNA **11** with a C3-linker in the G position of the CpG dinucleotide induced cytokine levels similar to those of background levels at 1.0 μg/mL concentration (Table 2). In in vivo experiments, parent CpG DNA **10** caused an increase of about 32% in the spleen weights of BALB/c mice at a dose of 5 mg/kg compared with control mice injected with vehicle (PBS) (Table 2). The administration of CpG DNA **11** caused an increase of about 5% in spleen weights of BALB/c mice at the same dose. Together, these in vitro and in vivo studies suggest that the presence of a CpG dinucleotide is required for spleen cell proliferation, cytokine secretion, and spleen enlargement.

The other CpG DNAs **12**–**18** were also studied for their immunostimulatory activity, and the results are presented in Table 2 (for concentration-dependent cytokine data see Supplementary Table 1). These data suggest that linker substitutions within two nucleotides away from the CpG dinucleotide either in the 5′- (**12**) or the 3′-flanking sequence (**16**) resulted in a signifi-

Table 1. Sequences of CpG DNA Showing the Position of Substitution

CpG DNA number	sequence (5′ → 3′)[a]
10	CCTACTAGCGTTCTCATC
11	CCTACTAGC**2**TTCTCATC
12	CCTACT**2**GCGTTCTCATC
13	CCTA**2**TAGCGTTCTCATC
14	CCT**22**TAGCGTTCTCATC
15	**22**TAGTAGCTTCTCATC
16	CCTACTAGCGT**2**CTCATC
17	CCTACTAGCGTTC**2**CATC
18	CCTACTAGCGTTC**22**ATC
19	CCT**6**CTAGCGTTCTCATC
20	CCTACTAGCGTTC**6**CATC
21	CCT**7**CTAGCGTTCTCATC
22	CCTACTAGCGTTC**7**CATC
23	CTATCTGACGTTCTCTGT
24	CTAT**1**TGACGTTCTCTGT
25	CTA**1**CTGACGTTCTCTGT
26	CTATCTG**2**CGTTCTCTGT
27	CTATC**2**GACGTTCTCTGT
28	CTA**2**CTGACGTTCTCTGT
29	**22222**TGACGTTCTCTGT
30	**2222**TGACGTTCTCTGT
31	**222**TGACGTTCTCTGT
32	**22**TGACGTTCTCTGT
33	**2**TGACGTTCTCTGT
34	CTAT**3**TGACGTTCTCTGT
35	CTA**3**CTGACGTTCTCTGT
36	CTA**33**TGACGTTCTCTGT
37	**33**TGACGTTCTCTGT
38	CTAT**4**TGACGTTCTCTGT
39	CTA**4**CTGACGTTCTCTGT
40	CTA**44**TGACGTTCTCTGT
41	**44**TGACGTTCTCTGT
42	CTAT**5**TGACGTTCTCTGT
43	CTA**5**CTGACGTTCTCTGT
44	CTA**55**TGACGTTCTCTGT
45	**55**TGACGTTCTCTGT
46	CTA**6**CTGACGTTCTCTGT
47	CTATCTGACGTTC**6**CTGT
48	CTA**7**CTGACGTTCTCTGT
49	CTATCTGACGTTC**7**CTGT
50	CTATCTG**8**CGTTCTCTGT
51	CTATCT**8**ACGTTCTCTGT
52	CTATC**8**GACGTTCTCTGT
53	CTAT**8**TGACGTTCTCTGT
54	CTA**8**CTGACGTTCTCTGT
55	CTATCTGACG**8**TCTCTGT
56	CTATCTGACGT**8**CTCTGT
57	CTATCTGACGTT**8**TCTGT
58	CTATCTGACGTTC**8**CTGT
59	CTATCTG**9**CGTTCTCTGT
60	CTATCT**9**ACGTTCTCTGT
61	CTA**9**CTGACGTTCTCTGT
62	CTATCTGACGT**9**CTCTGT
63	CTATCTGACGTTC**9**CTGT

[a] See Figure 2 for the chemical structures of substitutions **1**–**9**. All CpG DNAs are phosphorothioate backbone modified.

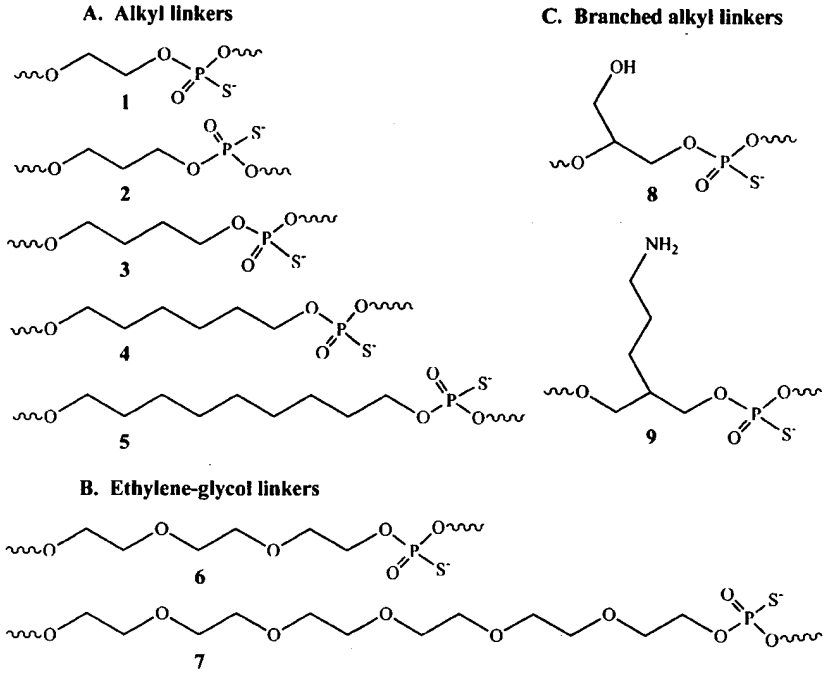

Figure 2. Chemical structures of non-nucleoside linkers used in the current study.

Table 2. Immunostimulatory Activity of CpG DNAs in BALB/c Mouse Spleen Cell Cultures and Splenomegaly in BALB/c Mice

CpG DNA number[a]	spleen cell proliferation (PI)[b]	cytokine, pg/mL[c]		spleen weight (mg)[d]
		IL-12	IL-6	
10	1.7 ± 0.27	1391 ± 179	6644 ± 380	130 ± 8
11	0.3 ± 0.02	119 ± 9	73 ± 10	105 ± 15
12	1.2 ± 0.1	491 ± 38	5435 ± 236	159 ± 18
13	2.1 ± 0.1	1709 ± 189	16105 ± 1767	211 ± 10
14	1.8 ± 0.1	2356 ± 191	15575 ± 814	230 ± 12
15	1.4 ± 0.1	1090 ± 50	6198 ± 682	203 ± 18
16	0.3 ± 0.02	157 ± 29	123 ± 62	90 ± 13
17	1.6 ± 0.2	1170 ± 59	5500 ± 673	170 ± 17
18	2.1 ± 0.3	2205 ± 182	10405 ± 1889	170 ± 12
M/V	0.2 ± 0.06	85 ± 23	59 ± 16	106 ± 17
LPS	2.6 ± 0.8	–	–	–

[a] See Table 1 for sequence, nature, and position of modification incorporated. [b] At 0.3 μg/mL concentration of CpG DNA. [c] Cytokine secretion in 24 h cell cultures at 1.0 μg/mL concentration of CpG DNA and [d] at 5 mg/kg dose, spleen weights measured after 48 h, and each value is an average of three or four mice.

cantly lower immunostimulatory activity. As the linker is moved away from the CpG dinucleotide (**13** and **17**), an increased immunostimulatory activity was observed, as determined in splenomegaly assays (Table 2). However, in spleen cell culture assays, substitution in the 5′-flanking sequence (**13**) caused an increase in spleen cell proliferation and IL-12 and IL-6 secretion (Table 2). CpG DNA **17**, which had the same modification substituted in the 3′-flanking sequence, caused a spleen cell proliferation and IL-12 and IL-6 secretion similar to that of the parent CpG DNA **10** (Table 2). The results of CpG DNAs **14**, **15**, and **18** suggested that substitution of two linkers did not result in an additive effect on immunostimulatory activity. More importantly, either single- or double-linker substitutions away from the CpG dinucleotide did not neutralize the immunostimulatory activity (Table 2). These results suggest that a nucleobase and a sugar ring are not required in the flanking sequences at least four to seven nucleotides away from the CpG dinucleotide for immunostimulatory activity. The data in Table 2 suggest that certain components in the CpG DNA are not required for its recognition by the receptors. In fact, the deletion of nucleobases as well as sugar rings at these nucleoside positions facilitates better recognition and/or interaction of modified CpG DNA by receptors and thereby potentiates immunostimulatory activity.

To examine if maintenance of proper interphosphate distance is required for immunostimulatory activity, ethyleneglycol-linkers (**6**, **7**) were incorporated in CpG DNA (**19**–**22**), and these modified CpG DNAs were examined for their immunostimulatory activity. The data presented in Table 3 (also see Supplementary Table 2 for concentration-dependent cytokine data) suggest that the substitution of either a tri- (**6**) or hexa- (**7**) ethyleneglycol-linker did not have a detrimental effect on the immunostimulatory activity of CpG DNA. However, substitution in the 5′-flanking sequence resulted in potentiation of the immunostimulatory activity compared with substitutions in the 3′-flanking sequence. Taken together, the results of in vitro and in vivo studies suggest that the substitution in the 3′-flanking sequence has a minimal effect on immunostimulatory activity compared with substitutions in the 5′-flanking sequence.

Table 3. Immunostimulatory Activity of CpG DNAs in BALB/c Mouse Spleen Cell Cultures and Splenomegaly in BALB/c Mice

CpG DNA number[a]	Cytokine, pg/mL[b]		spleen weight (mg)[c]
	IL-12	IL-6	
10	1391 ± 179	6644 ± 380	130 ± 8
19	2071 ± 44	15640 ± 887	275 ± 13
20	1189 ± 119	3921 ± 268	155 ± 9
21	1518 ± 148	14520 ± 825	263 ± 19
22	1151 ± 386	4046 ± 721	164 ± 22
M/V	85 ± 23	59 ± 16	106 ± 17

[a] See Table 1 for sequence, nature, and position of modification incorporated. [b] Cytokine secretion in 24 h cell cultures at 1.0 μg/mL concentration of CpG DNA and [c] at 5 mg/kg dose, and each value is an average of three or four mice.

Table 4. Splenomegaly Induced by CpG DNA Containing a C3-Alkyl-Linker in BALB/c Mice

CpG DNA number[a]	spleen weight (mg)[b]
23	121 ± 16
26	120 ± 14
27	228 ± 19
28	248 ± 20
vehicle	102 ± 8

[a] See Table 1 for sequences and position of modification. [b] Average spleen weight for four mice at a dose of 5 mg/kg.

Effect of C2−C9-Linker Substitutions in CpG DNA. To examine the effect of the length of an alkyl-linker on immunostimulatory activity, linkers of different carbon chain lengths, C2 to C9 (**1**−**5**), were incorporated in parent CpG DNA **23**, which had a "GACGTT" hexameric sequence, and these compounds were tested for their immunostimulatory activity. Since the preceding experiments had shown that incorporation of a C3-linker in the 5′-flanking sequence had a greater effect than it did in the other positions, we incorporated linkers **1**−**5** only in the 5′-flanking sequence to the CpG dinucleotide as shown in Table 1 to determine the optimal linker for potentiation of immunostimulatory activity.

The data from the splenomegaly assay for CpG DNAs **26**−**28** containing a C3-linker (**2**) at different nucleotide positions in the 5′-flanking sequence to the CpG dinucleotide are shown in Table 4. The parent CpG DNA **23** at a dose of 5 mg/kg caused an increase of about 21% in spleen weight compared with vehicle (PBS) control. At the same dose, CpG DNA **26**, which had a C3-linker adjacent to CpG dinucleotide in the 5′-flanking sequence, caused spleen enlargement similar to that caused by the parent CpG DNA. In contrast, a C3-linker placed two or five nucleotides away from CpG dinucleotide in the 5′-flanking sequence (CpG DNAs **27** and **28**) caused extensive splenomegaly (Table 4). These results are in agreement with the results observed with CpG DNAs **12** and **13**.

To evaluate the optimal linker size for potentiation of immunostimulatory activity, we measured IL-12 and IL-6 secretion induced by modified CpG DNAs in BALB/c mouse spleen cell cultures. All CpG DNAs induced concentration-dependent IL-12 and IL-6 secretion. Figure 3 shows data obtained at 1 μg/mL concentration of selected CpG DNAs, **25**, **28**, **35**, **39**, and **43**, which had a linker at the fifth nucleotide position in the 5′-flanking sequence to the CpG dinucleotide compared with the parent CpG DNA. The CpG DNAs, which contained C2- (**1**), C3- (**2**), and C4-linkers (**3**), induced

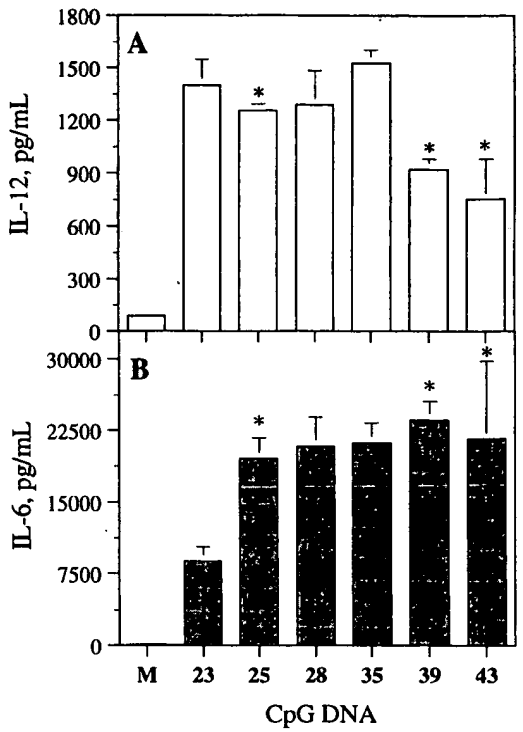

Figure 3. Cytokine (IL-12 and IL-6) secretion induced by CpG DNAs with a "GACGTT" hexameric motif containing a C2- (**25**), C3- (**28**), C4- (**35**) C6- (**39**), or C9-linker (**43**). M stands for media control. BALB/c mice spleen cell cultures were incubated with 1.0 μg/mL concentration of CpG DNAs for 24 h, and the levels of cytokines IL-12 (A) and IL-6 (B) secreted were measured as described in the Experimental Section. Each value is an average of three replicate samples. * indicates $p < 0.05$.

secretion of IL-12 production similar to that of the parent CpG DNA **23**. The CpG DNA that contained C6 and C9-linkers (**4** and **5**) at the fifth nucleotide position from CpG dinucleotide in the 5′-flanking sequence induced lower levels of IL-12 secretion than did the parent CpG DNA (Figure 3A), suggesting that substitution of linkers longer than a C4-linker results in the induction of lower levels of IL-12. All five CpG DNAs, which had linkers, induced 2−3 times higher IL-6 secretion than did the parent CpG DNA. The presence of a linker in these CpG DNAs showed a significant effect on the induction of IL-6 compared with CpG DNAs that did not have a linker. However, we did not observe length-dependent linker effect on IL-6 secretion.

Effect on Immunostimulatory Activity of CpG DNA Containing Ethyleneglycol-Linkers. We synthesized CpG DNAs **46** and **47**, in which a triethyleneglycol-linker (**6**) is incorporated at the fifth nucleotide position in the 5′- and at the fourth nucleotide position in the 3′-flanking sequences to the CpG dinucleotide, respectively. Similarly, CpG DNAs **48** and **49** contained a hexaethyleneglycol-linker (**7**) in the 5′- or the 3′-flanking sequence to the CpG dinucleotide, respectively. All four modified CpG DNAs (**46**−**49**) were tested in BALB/c mouse spleen cell cultures for cytokine induction (IL-12, IL-6, and IL-10) in comparison with parent CpG DNA **23**. All CpG DNAs induced concentration-dependent cytokine production over the concentration range tested (0.03−10.0 μg/mL) (Supplementary Table 3). The levels of cytokines induced at 0.3 μg/mL con-

Table 5. Cytokine Secretion Induced by CpG DNAs Containing an Ethyleneglycol-Linker in BALB/c Mice Spleen Cell Cultures

CpG DNA number[a]	cytokine, pg/mL[b]		
	IL-12	IL-6	IL-10
23	1887 ± 233	2130 ± 221	86 ± 14
46	2106 ± 143	2362 ± 166	78 ± 21
47	1888 ± 259	1082 ± 25	47 ± 14
48	2066 ± 153	2507 ± 66	73 ± 17
49	1318 ± 162	476 ± 13	25 ± 5
Medium	84 ± 13	33 ± 6	2 ± 1

[a] See Table 1 for sequence, nature, and position of modification incorporated. [b] Cytokine secretion in 48 h cell cultures at a concentration of 0.3 μg/mL of CpG DNA.

centration of CpG DNAs **46**–**49** are shown in Table 5. CpG DNAs **46** and **48**, which had an ethyleneglycol-linker in the 5′-flanking sequence, induced higher levels of IL-12 (2106 ± 143 and 2066 ± 153 pg/mL) and IL-6 (2362 ± 166 and 2507 ± 66 pg/mL) secretion than did parent CpG DNA **23** (Table 5). At the same concentration, **46** and **48** induced slightly lower levels of IL-10 secretion than did the parent CpG DNA (Table 5). CpG DNA **47**, which had a shorter ethyleneglycol-linker (**6**) in the 3′-flanking sequence, induced IL-12 secretion similar to that of the parent CpG DNA, but significantly lower levels of IL-6 and IL-10 (Table 5). CpG DNA **49**, which had a longer ethyleneglycol-linker (**7**), induced significantly lower levels of all three cytokines tested compared with the parent CpG DNA (Table 5).

Though triethyleneglycol-linker (**6**) had a chain length similar to that of C9-linker (**5**), the CpG DNA containing triethyleneglycol-linker had better immunostimulatory activity than did CpG DNA containing C9-linker, as determined by induction of cytokine secretion in spleen cell cultures. These results suggest that the lower immunostimulatory activity observed with CpG DNA containing longer alkyl-linkers (**4** and **5**) may not be related to their increased length but to their hydrophobic characteristics. This observation prompted us to examine substitution of branched alkyl-linkers containing hydrophilic functional groups on immunostimulatory activity.

Effect on Immunostimulatory Activity of CpG DNA Containing Branched Alkyl-Linkers. Two branched alkyl-linkers containing a hydroxyl (**8**) or an amine (**9**) functional group were incorporated in parent CpG DNA **23**, and the effects on immunostimulatory activity of the resulting modified CpG DNAs (**50**–**63**, Table 1) were examined. The data obtained with CpG DNAs **59**–**63**, containing amino-linker **9** at different nucleotide positions, in BALB/c mouse spleen cell cultures (proliferation) and in vivo (splenomegaly) are shown in Table 6.

Parent CpG DNA **23** showed a proliferation index of 3.7 ± 0.8 at a concentration of 0.1 μg/mL. At the same concentration, modified CpG DNAs **60**–**63** containing amino-linker **9** at different positions caused higher spleen cell proliferation than did the parent CpG DNA (Table 6). As observed with other linkers, when the substitution was placed adjacent to CpG dinucleotide (**59**), a lower proliferation index was noted compared with parent CpG DNA (Table 6), further confirming that the placement of a linker substitution adjacent to CpG dinucleotide has a detrimental effect on immunostimulatory activity. In general, substitution of an amino-

Table 6. Spleen Cell Proliferation Induced by CpG DNA Containing an Aminobutyryl Propanediol-Linker in BALB/c Mice Spleen Cell Cultures and Splenomegaly in BALB/c Mice

CpG DNA number[a]	spleen cell proliferation (PI)[b]	spleen weight (mg)[c]
23	3.7 ± 0.8	121 ± 16
59	2.5 ± 0.6	107 ± 11
60	9.2 ± 0.7	169 ± 16
61	8.8 ± 0.4	220 ± 8
63	7.8 ± 0.04	177 ± 12
M/V	1.2 ± 0.3	102 ± 8
LPS	2.8 ± 0.5	ND

[a] See Table 1 for sequences and position of modification. [b] At 0.1 μg/mL concentration in 48 h cell cultures. [c] Average spleen weight for four mice at a dose of 5 mg/kg; ND stands for not determined.

linker for 2′-deoxyribonucleoside in the 5′-flanking sequence (**60** and **61**) resulted in higher spleen cell proliferation than found with the substitution in the 3′-flanking sequence (**62** and **63**). Similar results were observed in the splenomegaly assay (Table 6), confirming the results observed in spleen cell cultures. Modified CpG DNAs containing glycerol-linker (**8**) showed immunostimulatory activity similar to or slightly higher than that observed with modified CpG DNA containing amino-linker (**9**) (data not shown).

To compare the immunostimulatory effects of CpG DNA containing linkers **8** and **9**, we selected CpG DNAs **54** and **61**, which had substitution in the 5′-flanking sequence and assayed their ability to induce cytokines IL-12 and IL-6 secretion in BALB/c mouse spleen cell cultures. Both CpG DNAs **54** and **61** induced concentration-dependent cytokine secretion. Figure 4 shows the levels of IL-12 and IL-6 induced by **54** and **61** in mouse spleen cell cultures at 0.3 μg/mL concentration compared with parent CpG DNA **23**. Both CpG DNAs induced higher levels of IL-12 and IL-6 than did parent CpG DNA **23**. CpG DNA containing glycerol-linker (**8**) induced slightly higher levels of cytokines (especially IL-12) than did CpG DNA containing amino-linker (**9**) (Figure 4). These results further confirm that the linkers containing hydrophilic groups are more favorable for the immunostimulatory activity of CpG DNA.

Effect on Immunostimulatory Activity of CpG DNA Containing Multiple Linker Substitutions. We examined two different aspects of multiple linker substitutions in CpG DNA. In one set of experiments, we kept the length of nucleotide sequence to 13-mer and incorporated one to five C3-linker (**2**) substitutions at the 5′-end (**29**–**33**). These modified CpG DNAs permitted us to study the effect of an increase in the length of linkers without causing solubility problems. In the second set of experiments, we incorporated two of the same linker substitutions (**3**, **4**, or **5**) in adjacent positions in the 5′-flanking sequence to the CpG dinucleotide to study if there would be any additive effect on immunostimulatory activity.

Modified CpG DNAs were studied for their ability to induce cytokine production in BALB/c mouse spleen cell cultures in comparison with parent CpG DNA **23**. All CpG DNAs induced concentration-dependent cytokine production. The data obtained at 1.0 μg/mL concentration of CpG DNAs is shown in Table 7. In this assay, parent CpG DNA **23** induced 967 ± 28 pg/mL of IL-12, 1593 ± 94 pg/mL of IL-6, and 14 ± 6 pg/mL of IL-10 secretion at 1.0 μg/mL of concentration. The data pre-

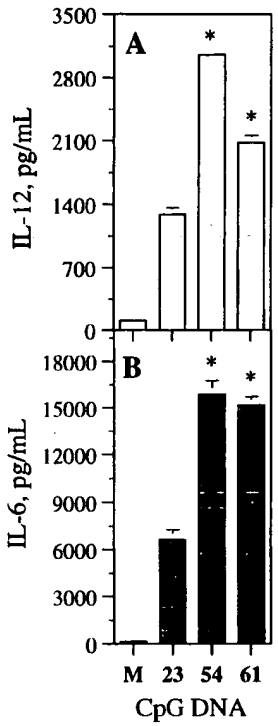

Figure 4. Cytokine (IL-12 and IL-6) secretion induced by CpG DNAs with a "GACGTT" hexameric motif containing a glyceryl- (**54**) or amino-propanediol-linker (**61**). M stands for media control. BALB/c mice spleen cell cultures were incubated with 1.0 μg/mL concentration of CpG DNAs for 24 h, and the levels of cytokines IL-12 (A) and IL-6 (B) secreted were measured as described in the Experimental Section. Each value is an average of three replicate samples. * indicates $p < 0.05$.

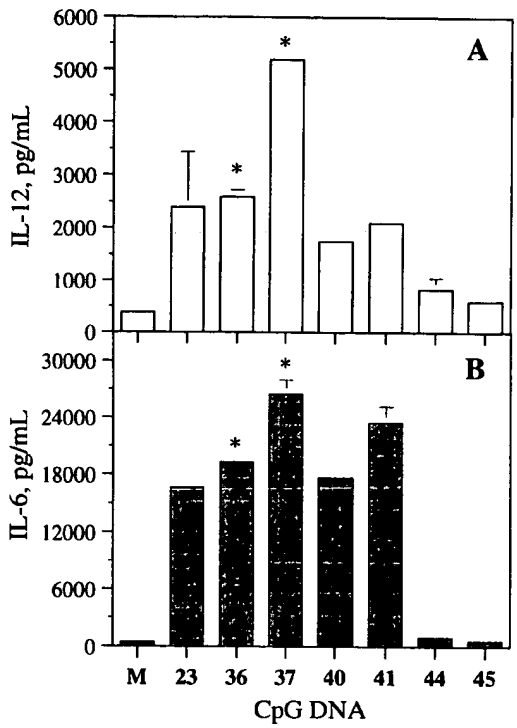

Figure 5. Cytokine (IL-12 and IL-6) secretion induced by CpG DNAs with a "GACGTT" hexameric motif containing two C4- (**36**, **37**) C6- (**40**, **41**), or C9-linker (**44**, **45**). M stands for media control. BALB/c mice spleen cell cultures were incubated with 1.0 μg/mL concentration of CpG DNAs for 24 h, and the levels of IL-12 (A) and IL-6 (B) secreted were measured as described in the Experimental Section. Each value is an average of three replicate samples. * indicates $p < 0.05$.

Table 7. Cytokine Secretion Induced by CpG DNA Containing Multiple C3-Linkers in BALB/c Mouse Spleen Cell Cultures

CpG DNA number[a]	cytokine (pg/mL)[b]		
	IL-12	IL-6	IL-10
23	967 ± 28	1593 ± 94	14 ± 6
29	1424 ± 325	3293 ± 478	36 ± 6
30	2336 ± 344	2409 ± 134	28 ± 1
31	1886 ± 142	2194 ± 184	36 ± 2
32	744 ± 164	3359 ± 706	45 ± 1
33	633 ± 255	3309 ± 337	58 ± 19
medium	126 ± 18	67 ± 8	5 ± 1

[a] See Table 1 for sequences, nature, and position of modification. [b] At a concentration of 1.0 μg/mL in 24 h cell culture.

sented in Table 7 suggest that as the number of linker substitutions decreased, IL-12 induction decreased. However, the induction of lower levels of IL-12 secretion by CpG DNAs **32** and **33** could be the result of the shorter length of CpG DNAs. Our studies with unmodified CpG DNA shorter than 15-nucleotides showed insignificant immunostimulatory activity (data not shown). Neither length nor the number of linker substitutions has a smaller effect on IL-6 secretion. Though IL-10 secretion increased with linker substitutions, the overall IL-10 secretion by these CpG DNAs was minimal.

CpG DNAs containing two linker substitutions (linker **3**−**36**; linker **4**−**40**; linker **5**−**44**) at the fourth and fifth positions in the 5′-flanking sequences to the CpG dinucleotide and the corresponding 5′-truncated versions **37**, **41**, and **45**, respectively, were tested for their ability to induce cytokine secretion in BALB/c mouse spleen cell cultures. The levels of IL-12 and IL-6 secreted at 1.0 μg/mL concentration are shown in Figure 5. The results presented in Figure 5 suggest that the immunostimulatory activity is dependent on the nature of the linker incorporated. The substitution of the fourth and fifth nucleosides with C4-linker **3** (CpG DNA **36**) had an insignificant effect on cytokine secretion compared with parent CpG DNA **23**, suggesting that the nucleobase and sugar ring at these positions are not required for receptor recognition and/or binding. The deletion of the nucleotides beyond the linker substitutions (CpG DNA **37**) caused higher IL-12 and IL-6 secretion than that found with CpG DNAs **23** and **36**. As expected, the substitution of two C6-linkers (**4**) resulted in IL-12 secretion lower than and IL-6 secretion similar to that induced by parent CpG DNA **23**. The 5′-truncated CpG DNA **41** induced higher cytokine secretion than did CpG DNA **40**. The CpG DNAs **44** and **45**, which had two C9-linkers (**5**), induced insignificant cytokine secretion, confirming the results obtained with monosubstituted CpG DNA containing the same linker as described above.

A number of first-generation CpG DNAs are currently being evaluated in clinical trials as monotherapies and in combination with vaccines against cancers and as adjuvants with vaccines and allergens for infectious diseases and allergies.[5,6,8,31,32] The current results indicate that the immunostimulatory activity of CpG DNAs can be modulated by incorporation of alkyl-linkers at appropriate positions in a CpG DNA molecule. These studies suggest that nucleotides in certain posi-

tions, especially in the 5′-flanking sequence to the CpG dinucleotide, are not significantly involved in recognition and/or interaction with the receptors. In fact, the deletion of a nucleoside at about three to six nucleotides away from the CpG dinucleotide in the 5′-flanking sequence potentiated immunostimulatory activity. The same modifications incorporated in the 3′-flanking sequence did not neutralize or increase the immunostimulatory activity compared with the parent CpG DNA, suggesting that perhaps only phosphate backbone, but not the sugar and nucleobase moieties, of the nucleotide portion involves in binding. In contrast, the CpG dinucleotide and the first two nucleotides adjacent to the CpG dinucleotide on both the 3′- and 5′-side play a critical role in immunostimulatory activity. Certain other modifications, however, are tolerated in the adjacent nucleotide positions to the CpG dinucleotide, as reported earlier.[24-26,28]

In another aspect, the linkers used in the study can be viewed as tethers that are joining two DNA fragments together. The lower immunostimulatory activity observed with shorter CpG DNAs (**33**) can be attained by attaching a short DNA fragment through the use of an appropriate tether (**28**). The results presented here with linker substitutions at the 5′-end of CpG DNA (**29**–**33**) suggest that these substitutions do not block the 5′-accessibility of CpG DNA for receptor recognition, in contrast to 5′-5′-linked CpG DNA.[26]

The CpG DNAs that contained C2-, C3-, and C4-linkers had similar immunostimulatory effects, suggesting that the length of the alkyl linker has an insignificant influence on recognition and/or binding of the modified CpG DNAs to the receptor(s). Similarly, tri- and hexa-ethyleneglycol-linkers also potentiated immunostimulatory activity when they were incorporated in the 5′-flanking sequence. However, the lower immunostimulatory activity observed with longer carbon linkers, such as C6 and C9, compared with shorter alkyl-linkers and long ethyleneglycol-linkers suggests that modifications that introduce increased hydrophobicity have adverse effect on immunostimulatory activity. It is important to note that within a CpG dinucleotide, a hydrophobic methyl substitution at the 5-position of cytosine neutralizes CpG-related immune stimulation[22] and a hydrophilic hydroxyl substitution at the same position retains immune stimulation.[27]

These studies suggest that while the presence of a CpG dinucleotide is critical for the observed immunostimulatory activity, modifications introduced in the 3′-flanking sequence have minimal effects on immunostimulatory activity. The introduction of nonnatural chemical modifications in oligonucleotides could contribute to increased nuclease stability and/or cellular uptake of oligonucleotides, which could result in altered immunostimulatory activity. In general, the 3′-exonucleases are mostly responsible for degradation of oligonucleotides in vivo. A number of studies have reported an increase in the in vivo stability of oligonucletides with appropriate chemical modifications incorporated at the 3′-end of the oligonucleotide.[33-36] In the present study, the alkyl-linker modifications incorporated toward the 3′-end should have imparted a relatively higher stability against nucleases than those incorporated toward the 5′-end; therefore, the former should have had a relatively greater immunostimulatory activity. In contrast, the results presented here show that CpG DNAs that have alkyl-linker substitutions in the 5′-flanking sequence (at least three to six nucleotides away from the CpG dinucleotide) had greater immunostimulatory activity than did the CpG DNAs that had substitutions in the 3′-flanking sequence. These results suggest that the observed increase in immunostimulatory activity was not the result of increased nuclease stability, but the result of the structural modifications introduced in the CpG DNA.

In addition, modified CpG DNAs containing different alkyl-linkers may have slightly different cellular uptake properties, depending on the nature of the linker incorporated (for example alkyl-linkers vs ethyleneglycol-linkers). CpG DNAs that contained the same kind of linker at different nucleotide positions should have possessed similar cellular uptake properties (for example, a C3-linker). We observed in the experiments reported here, however, that the immunostimulatory activity varied depending on the position of substitution within the CpG DNA sequence. These results suggest that the observed differences in the immunostimulatory activity could be the result of change in the required sequence (modifications incorporated within and adjacent to the CpG dinucleotide) and/or structural changes caused because of the modifications introduced (substitutions incorporated in the 5′- and 3′-flanking sequences), but not as a result of different cellular uptake properties.

In conclusion, the substitution of certain nucleotides with alkyl-linkers in the 5′-flanking sequence to the CpG dinucleotide potentiated immunostimulatory activity. Interestingly, the same substitution in the 3′-flanking sequence did not affect immunostimulatory activity compared with parent CpG DNA. While a C3-linker optimally potentiated immunostimulatory activity, longer ethyleneglycol- and branched alkyl-linkers also showed increased immunostimulatory activity. The linker substitutions in the 5′-flanking sequence increased IL-6 secretion several fold over that seen with unmodified CpG DNA. The ability of modified CpG DNAs to induce IL-12 secretion similar to that of parent CpG DNA is sufficient to skew the resulting immune response toward Th1 phenotype; the secretion of higher levels of IL-6 could be of an advantage for maturation of B-cells and rapid onset of T-cell specific humoral immune responses, when they are used as adjuvants with vaccines, allergens, and mAbs. It is not clear whether the differences observed in the immunostimulatory activity of the modified CpG DNAs resulted from altered recognition/binding events with the receptor(s) or initiation of different upstream signaling and transcriptional events compared with the parent CpG DNA. The ongoing studies of modified CpG DNA with specific immune-cell lineages should help us to understand the molecular mechanisms of interactions in detail and enable us to further fine-tune the incorporation of modifications, eventually allowing the broad application of CpG DNAs as immunological tools and therapeutic agents.

Experimental Section

CpG DNA Synthesis and Purification. CpG DNA were synthesized using β-cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem's 8909 Expedite DNA synthesizer on

1−2 μmol scale. The phosphoramidites of dA, dG, dC, and T were obtained from PE Biosystems. The DMT-protected phosphoramides of different linkers used in the study were obtained from Glen Research or ChemGenes. Beaucage's reagent was used as an oxidant to obtain phosphorothioate backbone modification.[37] After the synthesis, CpG DNAs were deprotected using standard protocols, purified by HPLC, and dialyzed against USP quality sterile water for irrigation (Braun). The CpG DNAs were lyophilized and dissolved again in distilled water, and the concentrations were determined by measuring the UV absorbance at 260 nm. All the CpG DNAs synthesized were characterized by CGE and MALDI-TOF mass spectrometry (Brucker Proflex III MALDI-TOF mass spectrometer with 337 nm N_2 laser) for purity and molecular mass, respectively. The molecular weights of representative CpG DNAs as determined by MS are (calculated; found): **10** (5658; 5660), **11** (5466; 5468), **12** (5482; 5484), **13** (5506; 5508), **14** (5331; 5333), **15** (5355; 5357), **16** (5491; 5493), **17** (5491; 5493), **18** (5340; 5342), **19** (5557; 5550), **21** (5589; 5584), **23** (5704; 5702), **24** (5539; 5626), **26** (5528; 5530), **27** (5537; 5539), **28** (5537; 5539), **34** (5567; 5565), **39** (5580; 5578), **43** (5622; 5620), **46** (5612; 5610), **48** (5744; 5740), **53** (5569; 5568), and **62** (5609; 5606). The purity of full length CpG DNAs ranged from 89−95%, as determined by CGE and the rest being n-1 and n-2. All CpG DNAs were synthesized and purified under identical conditions to minimize endotoxin contamination. In addition, CpG DNAs were tested for endotoxin contamination by Limulus assay (Bio-Whittaker) and endotoxin levels were <0.075 EU/mL.

Mouse Lymphocyte Proliferation Assay. Lymphocytes obtained from BALB/c mouse (4−8 weeks) spleens were cultured in RPMI complete medium as described earlier.[22,30] The cells were plated in 96-well dishes at a density of 10^6 cells/mL in a final volume of 100 μL. The CpG DNA, LPS (lipopolysaccharide, a positive control) (10 μg/mL), or medium was added to the cell culture in 10 μL of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at final concentrations of 0.1, 0.3, 1.0, and 3.0 μg/mL. The cells were then incubated at 37 °C. After 44 h, 1 μCi ^{3}H-uridine (Amersham) was added to the culture in 20 μL of RPMI medium, and the cells were pulse-labeled for another 4 h. The cells were harvested by automatic cell harvester, and the filters were counted by a scintillation counter. The experiments were performed two or three times for each CpG DNA in triplicate at each concentration. The averages were calculated, normalized, and presented as proliferation index.

Assays for IL-12, IL-6, and IL-10 Secretion in Mouse Spleen Cell Cultures. The secretion of IL-12, IL-6, and IL-10 in BALB/c mouse spleen cell cultures was measured by sandwich ELISA. The required reagents, including cytokine antibodies and cytokine standards, were purchased form PharMingen. ELISA plates (Costar) were incubated with appropriate antibodies at 5 μg/mL in PBSN buffer (PBS/0.05% sodium azide, pH 9.6) overnight at 4 °C and then blocked with PBS/10% FBS at 37 °C for 30 min. Cell culture supernatants and cytokine standards were appropriately diluted with PBS/10% FBS, added to the plates in triplicate, and incubated at 25 °C for 2 h. Plates were overlaid with 1 μg/mL appropriate biotinylated antibody and incubated at 25 °C for 1.5 h. The plates were washed extensively with PBS/0.05% Tween 20 and then further incubated at 25 °C for 1.5 h after the addition of streptavidine-conjugated peroxidase (Sigma). The plates were developed with Sure Blue Chromogenic reagent chromatin (Kirkegaard and Perry), and the color change was measured on a Ceres 900 HDI Spectrophotometer (Bio-Tek Instruments). The levels of IL-12, IL-6, and IL-10 in the cell culture supernatants were calculated from the standard curve constructed under the same experimental conditions for IL-12, IL-6, and IL-10, respectively.

Mouse Splenomegaly Assay of CpG DNA. Female BALB/c mice (4−6 weeks, 19−21 gm) were divided into different groups with three or four mice in each group. CpG DNA were dissolved in sterile PBS and administered intraperitoneally to mice at a dose of 5 mg/kg. After 72 h, mice were sacrificed, and the spleens were harvested and weighed.

Statistical Analysis. All results are presented as mean ± SD. The unpaired Student's t-test was used to determine the statistical significance of representative CpG DNAs in cytokine assays for any difference in immunostimulatory activity between the modified and parent CpG DNAs (Statpak, v 4.1).

Supporting Information Available: Tables of immunostimulatory activity and cytokine secretion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Exhibit 4

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Synthesis of oligodeoxyribonucleotides containing degenerate bases and their use as primers in the polymerase chain reaction

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ABSTRACT

Heptadecaoligodeoxyribonucleotides containing one or more of the bases, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (P), 2-amino-6-methoxyaminopurine (K), and hypoxanthine (I) and combinations of P with K and I have been synthesised on a DNA synthesiser. The stability of duplexes containing these base-m dified oligomers with P/A, P/G, K/C and K/T; P/A, P/G, I/C, I/T and I/A, I/G, I/C, I/T base pairs were compared by measuring their melting transition (T_m) values. Oligomers containing both P and K and P and I were more stable than those with I alone or with mismatches. These oligomers together with one with a P base at the 3'-end were used as primers in polymerase chain reaction (PCR) experiments. They were all effective primers except one with I alone and a triple mismatch. Thus the use of the degenerate bases P and K in primer design is established.

INTRODUCTION

In recent publications we reported separately the synthesis of monomers containing the degenerate bases P, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one and K, 2-amino-6-methoxyamine purine.[1,2] Due to the ability of both bases to exist in their amino and imino tautomers, they can potentially base pair with both A and G, and C and T respectively (see Figure 1). We found that oligomers containing one or more P bases formed DNA duplexes of comparable stability to the parent duplexes and also showed sharp transitions on melting. However, the purine analogue K formed somewhat less stable duplexes. Further evidence from NMR spectroscopy of the octamers d(CGAATPCG)$_2$ and d(CGGATPCG)$_2$ confirmed that the base pairs P/A and P/G were essentially of the Watson-Crick type.[3,4]

We also discussed the potential use of these bases in hybridisation probes and primers when either or both of these bases can be put at positions of degeneracy thus either avoiding the need for multiple-chain primers (or probes) or significantly reducing the chain multiplicity. Indeed oligonucleotides containing several P bases were effective in dot blot hybridisation and DNA sequencing experiments.[5] The most common methods applied in probing gen mic r copy DNA based on protein amino acid sequences are the use of mixed probes to cover all possible codon assignments[6,7] or by inserting the 'universal' base hypoxanthine (I)[8,9,10] at sites of degeneracy .The first method is successful when the multiplicity is not too high. However machine synthesis of high multiplicity probes is probably not perfect and in any case it is not possible to check whether the single perfect sequence is present in the mixture. Moreover when hypoxanthine (I) is used the latter can base pair with low discrimination with all the bases; non specific hybridisation can occur and as a consequence result in a high background signal.[11] As for the PCR, mixed oligonucleotide primer amplification (MOPAC) technology is very often used, the rate of success usually depending on the primer complexity (up to 1024 has been used successfully). Increased primer complexity is associated with an increase in non specific priming.[12,13] In this paper we describe the synthesis of oligomers containing both bases P and K, P and I and I alone. The stability of these base modified oligomers are compared with the perfect complementary primers and those with mismatches in PCR reactions . As far as we are aware, it is the first time that degenerate bases such as P and K have been used for PCR amplification. To confirm that recognition of base P in the primer by the *taq* polymerase enzyme leads to chain extention, an oligomer with P at the 3'-end was synthesised. The potential use of bases P and K and P and I in primers, as a way of largely reducing multiplicity without background enhancement, will be discussed.

MATERIALS AND METHODS

Phosphoramidite monomers P and K were synthesised as described previously[1,2] and the deoxyinosine monomer was from Applied Biosystems Inc.

Synthesis of functionalised controlled pore glass (CPG) support carrying the 3'-O-succinate of (5-O-dimethoxytrityl-2-deoxyribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c][1,2]oxazin-7-one

This was carried out by the method of Atkinson and Smith.[14] The nucleoside loading of the functionalised CPG was 57.2μm 1./g.

Oligonucleotide synthesis

Oligonucleotides (Tabl) were synthesised using an Applied Biosystem 380B instrument with the normal synthesis cycle. The CPG functionalised with the dimethoxytrityl derivative of the

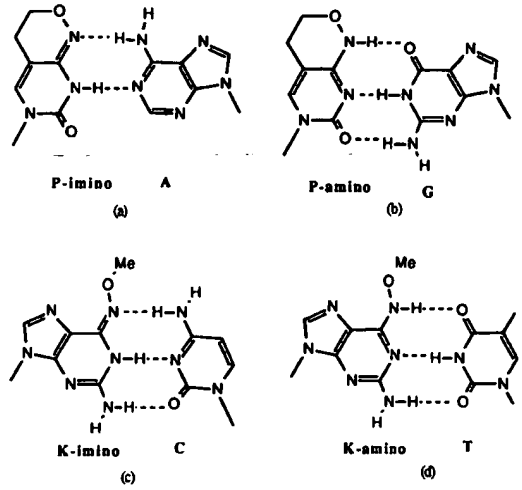

Figure 1. Pyrimidine analogue P in its imino (a) and amino (b) tautomeric form pairing with adenine and guanine. Purine analogue K in its imino (c) and amino (d) tautomeric form pairing with cytosine and thymine. Both analogues form Watson-Crick base pairs.

Table 1. Melting temperatures (T_m) of heptadecamer duplexes in 6×SSC buffer at pH 7.0.

		T_m(°C)			T_m(°C)
1.	ACTTGGCCACCATTTTG TGAACCGGTGGTAAAAC	72	7.	ACTTGICCICIATTTTG --------C---T-G-----A---	50
2.	ACTTGGCCGCCATTTTG TGAACCGGCGGTAAAAC	75	8.	ACTTGKCCKCPATTPTG --------C-----T--G------A---	55
3.	ACTTGGCCACCATTTTG --------T-------C--- C---------	43	9.	ACTTGICCICPATTPTG ---------C---T-G------A----	59
4.	ACITGICCACCITTITG ---A---C---------T---A---	57	10.	ACTTGICCICIATTTTG ---------C---C-G------A---	53
5.	ACPTGKCCACCKTTPTG -----A----C---------T----A----	60	11.	ACTTGKCCKCPATTPTG ---------C-----C--G------A----	55
6.	ACPTGICCACCITTPTG ----A----C---------T---A----	60	12.	ACTTGICCICPATTPTG ---------C---C--A-----A---	63

nucleoside P was used to provide an oligonucleotide having the P nucleoside at the 3'-end of the oligomer.

Deprotection was complete after treatment with aqueous NH_3 at 55°C overnight. Purification was carried out by h.p.l.c. on a Waters system using a Whatman Sax partisphere or a Hichrom partisil 10 sax column and a potassium phosphate (pH 6.3) gradient in aqueous 60% formamide.

Melting transitions (T_m) of oligonucleotide duplexes

Melting transitions were measured at 260nm in 6×SSC (0.9M sodium chloride, 0.09M sodium citrate, pH 7.0) buffer at an oligomer strand concentration of ~ 3μM. Absorbance vs. temperature for each duplex was obtained using a Perkin Elmer Lambda 2 spectrometer connected with a Peltier block and temperature programmer. The temperature was increased by 1°C/min. and melting temperatures (T_m) were determined as the maxima of the respective differential curves with an errror of ±1°C.

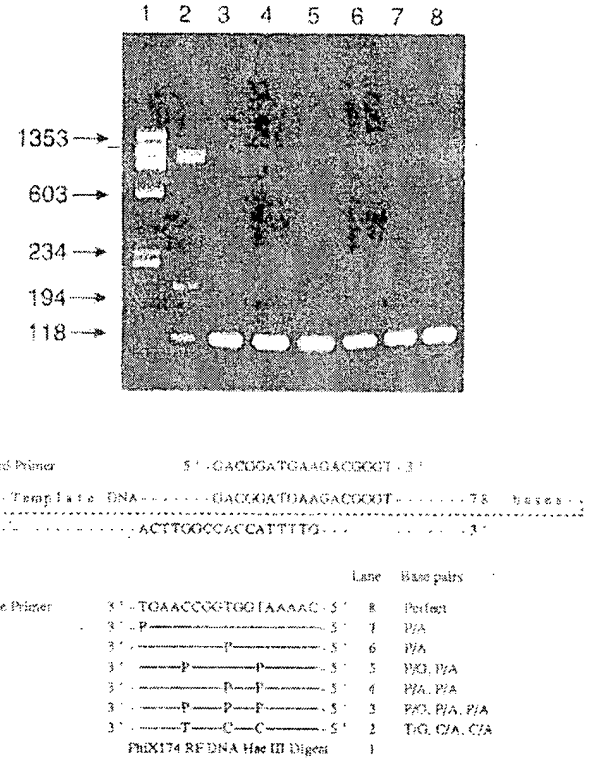

Figure 2. Thermal cycle was: denaturing at 92°C for 1 min, annealing at 44°C for 1 min, chain extension at 70°C for 1 min, final extension at 70°C for 5 min and the number of cycles = 30.

Polymerase chain reaction

Single stranded bacteriophage M13 DNA which contained an insert corresponding to the Tyr TS gene of *Bacillus. stearothermophilus* [15] was used as template. PCR reactions were done on a Techne programmable Dri Block PHC-1 apparatus. Each 100μl reaction contained 1μM template DNA, 200pm 1 f each primers, 50μM of each dNTP, 5 units of *Taq* polmerase in buffer (5μl) provided by Promega and 75μl of paraffin oil. The thermal cycle was: denaturing at 92°C, annealing at 36−44°C, chain extension at 62−70°C each for 1 min, final extension at 62−70°C for 5 min and number of cycles = 30. The regions of the template to be amplified are shown on Figures 2,3 and 4. After amplification the PCR products were electrophoretically separated on standard 2% agrose (BRL Inc.) minigels, containing ethidium bromide and photographed with *uv* illumination.

RESULTS AND DISCUSSION

Examination of the Table, shows the T_m (°C) values of 17-mer DNA duplexes in 6×SSC buffer. Each of them (entries 4−12) contains f ur modified bases and they should be compared with the respective native (entries 1 and 2) and mismatch (entry 3) duplexes . All the DNA duplexes (entries 4−12) showed higher T_ms than the one with three mismatches(entry 3) (δT_m=10−20°C). In all cases, sharp melting transiti ns were found indicating cooperativity in their thermal dissociati ns. Entries 4, 5 and 6 have modified bases at sites of T/A, G/C,

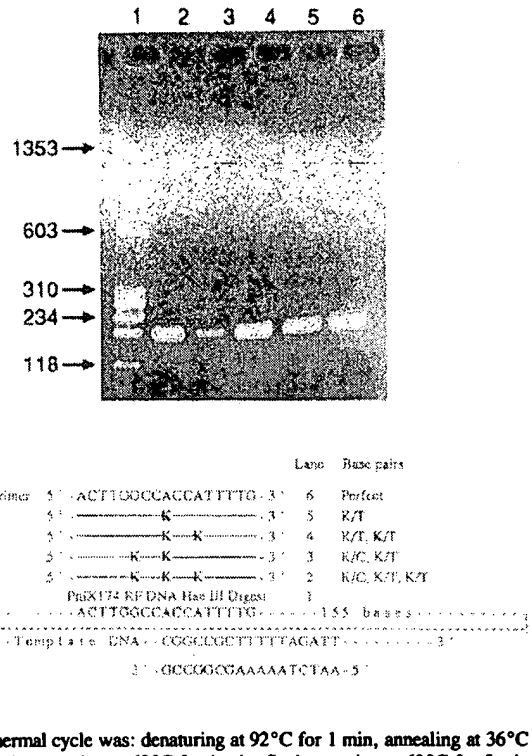

Figure 3. Thermal cycle was: denaturing at 92°C for 1 min, annealing at 36°C for 1 min, chain extension at 62°C for 1 min, final extension at 62°C for 5 min and the number of cycles = 30.

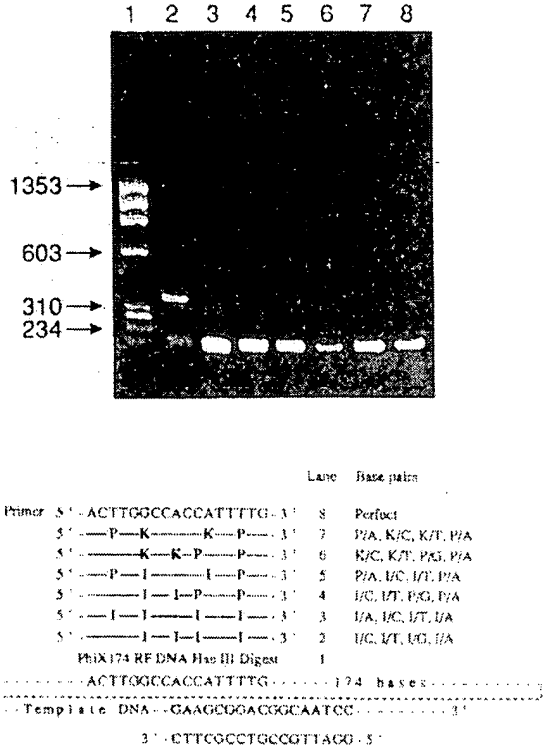

Figure 4. Thermal cycle was: denaturing at 92°C for 1 min, annealing at 36°C for 1 min, chain extension at 62°C for 1 min, final extension at 62°C for 5 min and the number of cycles = 30.

A/T and T/A base pairs and showed the following trend: P/A, I/C, I/T and P/A = P/A, K/C, K/T and P/A > I/A, I/C, I/T and I/A. The difference between entries 5, 6 and 4 is small (~3°C) probably related to the stability of I/A and I/C base pairs.[16,17] Duplexes 8 (K/C, K/T, P/G and P/A) and 9 (I/C, I/T, P/G, P/A) showed more stable duplexes (T_m= 55 and 59°C respectively) when compared with entry 7 (T_m= 50°C) which has I/T and I/A base pairs. However the duplex in entry 12 with I/C, I/C, P/G and P/A pairs showed higher stability by 10°C than the corresponding one (entry 10) which contained solely I residues at these positions. The number of examples in the Table, while allowing relevent comparisons, is not sufficient to let firm conclusions to be drawn, particularly when relatively large sequence-related effects occur.[18] Nevertheless, the use of P as a pyrimidine analogue together with K or I as a purine may provide a more satisfactory solution to reduction in primer multiplicity. The PCR experiments provide clear evidence that this is so.

Figures 2, 3 and 4 show the results of a number of PCR experiments using primers containing P, K and I alone, P and K and P and I in combination. The template employed in these experiments was a single stranded M13 DNA which contained an insert corresponding to the Tyr TS gene of *Bacillus stearothermophilus*. In Figure 2, the forward primer was the perfect complement while all the reverse primers contained modified bases except for the positive control. It shows that all the primers with one or more P residues including the one with the P base at the 3' end (lane 7) give the PCR product of the correct length (112 base pairs). This confirms that the nzyme *taq* polymerase recognises the P base and chain extends from it. For this to happen the P base must form a Watson–Crick base pair with A (as indeed it does[3]) since a mismatch at the 3'-end is known to result in failed amplification.[19,20] It is worth mentioning that the primer with three mismatches (lane 2) resulted in non specific priming, more than one PCR product being observed. Primers with one, two and three K bases gave a single product (189 base pairs) (Figure 3) in this case the annealing temperature was 36°C in order to achieve amplification.The lower annealing temperature is in accord with the observed T_ms of these K containing duplexes.[2] Figure 4 shows that all the primers containing P and I, P and K and I only give the expected PCR product (208 base pairs) except that in lane 2; the latter primer with four I residues exhibits non specific priming and hence is not effective compared with corresponding primers containing P and K (lane 6) and P and I (lane 4).

CONCLUSIONS

We have shown in this work that duplexes containing P and K, and P and I have better stability than the corresponding duplexes with only I. In duplexes with P and K, these bases have degenerate properties but their lower T_m values compared with the parent duplexes is probably dependent on the *syn* oriented methoxyl group of the K base, interfering with the normal Watson–Crick base pairing. In th case of duplexes containing both P and I, the latter base can form pairs with C and weakly with T but its base stacking properties contribute t the duplex stability.[16] In th PCR experiments, it was shown that P and K and P and I combinations can be used successfully with less non

specific priming than when I is used alone. From the above results it is clear that effective primers can be designed with low multiplicity, avoiding the disadvantages of those containing I alone.

In preliminary experiments (Arizmendi et al.)[21] to investigate the use of the analogues in primers and hybridisation probes, the stategy of Walker and co-workers[22] was applied to the isolation of a genomic DNA clone of a subunit of the NADH ubiquinone oxidoreductase from bovine heart mitochondria; this had already been isolated with difficulty using fully mixed primers and probes. We found that forward and reverse primers both containing P and K (multiplicity reduction factor of 15) for PCR amplification of the bovine genomic DNA were as effective as fully mixed primers. Probes containing P and K and P and I (reduction factors 11 and 21 resp.) for the PCR amplification product gave much enhanced signals. Subsequent cloning and plaque hybridisation detection with these degenerate probes gave a high percentage of clones with the expected sequence.

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